GYNECOLOGIC ONCOLOGY 32, 273–277 (1989)

Antiproliferative Effect Of Gossypol and Its Optical Isomers on Human Reproductive Cancer Cell Lines[1]

VIMLA BAND,*·†·[2] ANITA P. HOFFER,‡ HAMID BAND,§·‖ ANN E. RHINEHARDT,*·† ROBERT C. KNAPP,*·† STEPHEN A. MATLIN,¶ AND DEBORAH J. ANDERSON*·†·§·‖

Divisions of Gynecologic Oncology and Immunogenetics,§ Dana Farber Cancer Institute, Departments of †Obstetrics and Gynecology, ‡ Anatomy and Urology, and ‖Pathology, Harvard Medical School, Boston, Massachusetts 02115, and ¶Department of Chemistry, The City University, Northampton Square, London, ECIV OHB, United Kingdom*

Received July 30, 1987

The antiproliferative effect of gossypol and its optical isomers on various human cell lines of reproductive and nonreproductive tissue origin was studied. Various reproductive cancer cell lines of ovarian, gestational, and testicular origin were highly sensitive (IC50 values of 0.86–1.98) to gossypol. The antiproliferative action of gossypol was not restricted to reproductive cancers, as nonreproductive cancer cell lines were also equally sensitive (IC50 values of 0.69–3.55). In addition, actively proliferating untransformed cells such as fibroblasts and PHA-activated lymphocytes were also sensitive (IC50 values of 0.87–2.51). (−)-Gossypol was 3.6–12.4 times more potent than (+)-gossypol and 1.48–2.65 times more potent than (±)-gossypol. The most sensitive indicator of gossypol action was a decrease in DNA synthesis followed by inhibition of protein synthesis and uptake of rhodamine-123 by mitochondria as tested in an ovarian cancer cell line (OVCA 433) and a fibroblast line (Hs27). These results indicate that gossypol possesses a general nonselective antiproliferative action toward human cells *in vitro*. Further, the pharmacologic activity of gossypol as an antiproliferative agent is primarily attributable to its (−) isomer, which is also the active isomer as a contraceptive. © 1989 Academic Press, Inc.

INTRODUCTION

Gossypol, a compound derived from the cotton seed, has been shown to interrupt reproductive functions in both men and women [1]. Based on its potent effect on spermatogenesis at low oral doses gossypol has been extensively used as a male contraceptive in China [1]. During the course of studies on the mechanism of action of gossypol as a selective spermicide, it was observed to be a potent inhibitor of the lactate dehydrogenase C4 enzyme of sperm [2]. As anaerobic metabolism is relatively higher in cancer cells [3], these observations prompted studies to test its effect on the proliferation of cancer cells. Indeed, several studies have demonstrated an *in vitro* antiproliferative effect of gossypol [3–5]. In addition, gossypol has also been shown to inhibit the growth of transplanted tumors *in vivo* [6]. As gossypol is known to have a rather selective activity on normal reproductive tissues, we wished to investigate whether it will exert any selective antiproliferative effect toward cancer cell lines of reproductive origin. Recently the optical isomers of gossypol have been isolated and it has been found that the *in vivo* contraceptive effect of (±)-gossypol is attributable to its content of (−) isomer [7,8], although the (+) isomer shows activity *in vitro* as a spermicide [9]. It is not, however, known whether the antiproliferative activity of the widely used racemic gossypol is intrinsic to the active isomer or represents an untoward action due to the noncontraceptive isomer. Therefore, in the present study we also compared the effects of racemic gossypol and the (−) and (+) isomers of gossypol on the proliferation of different human cell lines (both untransformed and cancer cell lines) of different tissue origins and mitogen-activated peripheral blood mononuclear cells. Our results show that gossypol exerts a general *in vitro* antiproliferative activity toward both reproductive and nonreproductive cancer cell lines, as well as toward untransformed proliferating cells. Further, the antiproliferative activity of gossypol, like its other major biological activities, is attributable to its (−) isomer.

[1] This study was supported by Centocor Grant 933003 and NIH Grant ROI CA 42738.

[2] To whom all correspondence should be addressed at Division of Cancer Genetics, Dana Farber Cancer Institute, 44 Binney Street, Boston, MA 02115.

0090-8258/89 $1.50

MATERIALS AND METHODS

Gossypol and isomers. (±)-Gossypol was prepared and purified from (±)-gossypol acetic acid and (+)-gossypol was isolated and purified from *Thepesia populnea* [7]. (−)-Gossypol was separated from (±)-gossypol as described previously [9].

Gossypol and its (−) and (+) isomers were freshly dissolved in dimethyl sulfoxide (DMSO; 10 mg/ml) and further diluted in medium.

Cell lines. Ovarian cell lines (OVCA 420, 429, 432, and 433) were established and kindly provided by Dr. Herbert Lazarus, Dana Farber Cancer Institute. WI-38 and Hs27 (fibroblast), Tera-2 (human embryonal carcinoma), WM9 (malignant melanoma), and SK-UT-1 (mesodermal tumor of uterus) cell lines were obtained from American Type Culture Collection, Rockville, Maryland. Other cell lines were generous gifts from the following. OVCAR 3 (ovarian carcinoma): Dr. Robert Ozols; BeWo and Jar (choriocarcinomas): Dr. R. A. Pattilo; 577M and 833K (testicular teratocarcinomas): Drs. Fraley and Bronson; HL-60 (promyelomonocytic leukemia): Dr. D. Kufe.

All the cell lines used in this study were free of mycoplasma (tested by Mycotect kit, Bethesda Research Laboratories, Gaithersberg, MD).

Cell Culture. Cells were grown in minimum essential medium (MEM) (OVCA 420, 429, 432, 433, SK-UT-1, WM9, and OVCAR 3), RPMI 1640 (TCA, 577M, JAR, BeWo, HL-60, and mononuclear cells), Dulbecco's modified Eagle's medium (DMEM) (Hs27 and WI-38), or McCoy's 5a medium (Tera-2), supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, 100 μg/ml streptomycin, 100 units/ml penicillin, and 1% nonessential amino acids (GIBCO Laboratories, Grand Island, NY). The adherent cells (all the cell lines except HL-60) were removed from the flasks by treatment with 0.25% trypsin + 0.02% EDTA in Hanks' balanced salt solution without calcium and magnesium (GIBCO) for 10–15 min at 37°C. Cells were washed twice and counted before plating.

Peripheral blood mononuclear cells were prepared by centrifuging heparinized blood on Ficoll–Hypaque (Nunc, Roskilde, Denmark) gradients. Cells at the interface were collected, washed, and plated at 10^6 cells/ml with 1 μg/ml final concentration of phytohemagglutinin (PHA; 3× crystallized, Wellcome Research Laboratories, Research Triangle Park, NC) for 72 hr.

Measurement of cell proliferation. Cells were plated overnight before the addition of gossypol. For measuring the effect on viability, 2.5×10^4 cells (except mononuclear cells which were used at 10^6/well) were plated/well into 24-well plates (Flow Laboratories, McLean, VA) in a final volume of 1 ml. After 72 hr treatment with various concentrations of gossypol or isomers, cells were harvested for viable (trypan blue excluding) cell counting.

For measurement of [^{3}H]thymidine incorporation, 5×10^3 cells (except mononuclear cells which were used at 2×10^5 cells/well) were plated per well in 96-well flat-bottomed plates (Flow Labs) in a final volume of 200 μl. Cells were treated with gossypol and isomers as described above. [*methyl*-^{3}H]Thymidine (8 μCi) (sp act 77.8 Ci/mmole, New England Nuclear, Boston, MA) was added for the last 12 hr. Cells were trypsinized and harvested onto glass fiber filters using a Titretek cell harvester (Model 530, Flow Labs). Radioactivity was counted in a scintillation counter (Beckman) using a toluene-based scintillation mixture (Betaflour, New England Nuclear).

Effect on DNA and protein synthesis. [^{3}H]Thymidine and [^{3}H]leucine incorporation were used as a measure of DNA and protein synthesis, respectively. Cells (4×10^4) were plated overnight in the wells of a 96-well plate and then incubated with gossypol or isomers for 6, 12, 24, 48, or 72 hr. [^{3}H]Leucine (1 μCi) (sp act 50 Ci/mmole) or [^{3}H]thymidine was added 1 hr before harvesting. Harvesting was done as described above.

Effect on rhodamine-123 (R-123) uptake. For R-123 uptake, 10^5 cells were plated overnight on glass coverslips inside 24-well plates and then treated with gossypol for 6, 12, 24, 48, or 72 hr. At the end of the incubation period cells were stained with R-123 (Eastman Kodak; 10 μg/ml in medium) for 20 min at 37°C. Cells were washed three times in medium before examination under phase contrast and fluorescence optics to determine the percentage of total cells showing positively stained (fluorescent) mitochondria [10]. At least 200 cells were counted per coverslip.

The results are expressed as means ± SD of three experiments each in triplicate.

RESULTS

Comparison of the antiproliferative effect of gossypol and its isomers. Antiproliferative effects of gossypol and isomers were assessed by counting the number of viable cells and incorporation of [^{3}H]thymidine into DNA. In all cell lines a complete dose response was performed using 0.312, 0.625, 1.25, 2.5, 5, 10, and 20 μg/ml of each isomer as well as the racemate. The controls were treated with the highest concentration of DMSO used as a solvent (this did not exceed 0.2%). This concentration of DMSO did not alter the thymidine incorporation or viable cell number significantly.

Figure 1 shows the relation between the log of the concentration of gossypol and the amount of [^{3}H]thymidine incorporated (A) or the % viable cells (B) in OVCA 433 cells as a representative example of the dose responses obtained. A dose-dependent inhibition of both the pa-

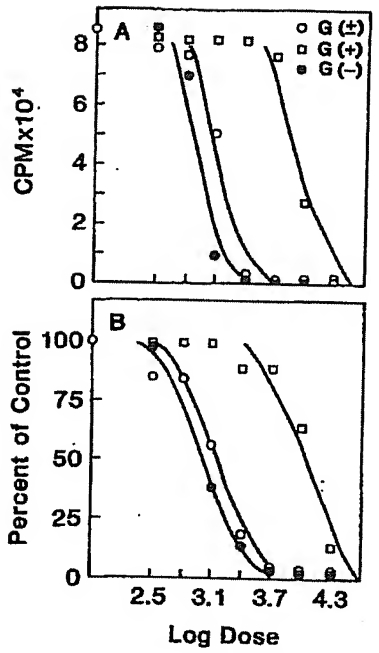

FIG. 1. Log relation of the effect of gossypol and its isomers on [^{3}H]thymidine incorporation (A) or cell viability (B). Ordinate represents percentage effect as compared to control which is taken as 100%. Cells were treated with gossypol for 72 hr. [^{3}H]Thymidine was added for last 12 hr. Best-fitting curves were drawn visually.

rameters was observed. The middle portions of the dose–response curves are linear and parallel to each other. In both cases (−)-gossypol is the most potent while (+)-gossypol shows the least activity. (±)-Gossypol shows intermediate activity and is about half as potent as (−)-gossypol.

Comparison of Figs. 1A and 1B shows a close correlation between the reduction of viable cell number and [^{3}H]thymidine incorporation by (±)-gossypol and its isomers. Similar results were observed with (±)-gossypol in all the other cell lines used in the present study (data not shown). Therefore, inhibition of thymidine incorporation was used as a measure of antiproliferative action of gossypol in subsequent experiments.

For comparing different isomers, the responses were expressed as a percentage of the control thymidine incorporation. The concentration of gossypol or isomers producing 50% inhibition of the thymidine incorporation (IC50) was determined by comparing visually drawn best-fitting curves.

As seen in Table 1 all the reproductive cancer cell lines tested were highly sensitive to gossypol with IC50 values in micromolar range. This effect is a general antiproliferative effect as cancer cell lines of nonreproductive tissue origin, untransformed fibroblast cell line, as well as PHA-activated peripheral blood mononuclear cells showed a similar pattern of sensitivity. All cells were more sensitive to (−)-gossypol as compared to (+) isomer (3.6- to 12.4-fold). (−)-Gossypol was 1.48- to 2.65-fold more active compared to the (±) form.

Effect of gossypol and isomers on DNA and protein synthesis, and on mitochondrial function in OVCA 433 cells. We further assessed the effect of (±)-gossypol and its isomers on two other important indicators of cellular metabolism, protein synthesis and mitochondrial function, both of which are known to be inhibited by gossypol [11]. OVCA 433 cell line was used in these experiments. The kinetics of inhibition of [^{3}H]leucine incorporation into proteins, and of R-123 uptake into mitochondria, were studied in comparison with inhibition of DNA synthesis. Three concentrations (0.312, 1.25, and 5 μg/ml) of each compound were tested at various time points (6, 12, 24, 48, and 72 hr). The effects were dose-related though the pattern of inhibition was similar at all the concentrations. For simplicity only the results obtained with 5 μg/ml concentration of the isomers are presented. As seen in Fig. 2, (−)-gossypol was clearly the most potent isomer on all the three parameters. The effect of (−) isomer also preceded that of (±) and (+) analogs on all these parameters.

A comparison of the effect of gossypol and its isomers on the OVCA 433 cell line revealed that the most sensitive indicator is DNA synthesis followed in order by protein synthesis and mitochondrial metabolism.

DISCUSSION

Recent reports have demonstrated that gossypol possesses *in vitro* antiproliferative activity at concentrations achievable during its use as a contraceptive in humans [3–5]. Considering the rather selective *in vivo* activity of gossypol toward reproductive tissues, it was of interest to investigate whether reproductive cancers show a higher sensitivity to the antiproliferative effects of gossypol. More recently, it has been shown that the *in vivo* antifertility action of gossypol, which consists of a racemic mixture of its optical isomers, is mostly attributable to its content of (−) isomer [7,8]. It was, therefore, of interest to see if the antifertility as well as antiproliferative and other actions of gossypol are due to the (−) isomer or whether the different isomers have divergent spectra of activities. To answer these questions we compared the racemic gossypol with (−) and (+) isomers of gossypol as antiproliferative agents. In addition, we analyzed their relative potencies to inhibit other cellular metabolic activities known to be blocked by gossypol.

Inhibition of thymidine incorporation correlated well with the direct assessment of cell viability when the isomers were tested on OVCA 433 cells and a fibroblast line (Hs27, data not shown). By this criterion the (−)

TABLE 1

Comparative Antiproliferative Activity of the Optical Isomers of Gossypol on Various Human Cell Lines *in Vitro*[a]

Cell lines	IC50 (μg/ml)			Relative potencies	
	G(±)	G(+)	G(−)	G(+)/G(−)	G(±)/G(−)
OVCA 420	1.98 ± 0.6	6.06 ± 0.9	0.85 ± 0.3	7.1	2.3
OVCA 429	1.25 ± 0.8	5.85 ± 3.5	0.47 ± 0.1	12.4	2.7
OVCA 433	1.28 ± 0.3	4.43 ± 3.3	0.62 ± 0.3	7.1	2.1
OVCA 432	1.24 ± 0.6	5.27 ± 2.3	0.57 ± 0.2	9.2	2.2
OVCAR 3	0.80 ± 0.2	3.01 ± 0.5	0.32 ± 0.0	9.4	2.5
833K	1.29 ± 0.1	5.37 ± 0.1	0.87 ± 0.0	7.3	1.5
Tera-2	1.16 ± 0.4	3.38 ± 1.0	0.49 ± 0.2	6.9	2.4
BeWo	1.09 ± 0.1	2.39 ± 0.1	0.44 ± 0.1	5.4	2.5
JAR	0.86 ± 0.7	2.72 ± 1.3	0.44 ± 0.3	6.2	2.0
HL-60	0.69 ± 0.0	1.66 ± 0.3	0.33 ± 0.0	5.0	2.1
WM9	3.55 ± 0.3	7.41 ± 1.7	1.62 ± 0.4	4.6	2.2
WI-38	0.87 ± 0.0	3.09 ± 0.1	0.48 ± 0.0	6.4	1.8
Hs27	1.43 ± 0.1	5.38 ± 1.6	0.54 ± 0.4	9.9	2.7
PHA-PBL	2.51 ± 0.2	4.90 ± 0.1	1.35 ± 0.4	3.6	1.9

[a] Mean IC50 values indicate the concentrations of various compounds that reduce the [^{3}H]thymidine incorporation by 50% as compared to controls after 72 hr treatment. [^{3}H]Thymidine was added for last 12 hr. Data are expressed as means ± SD.

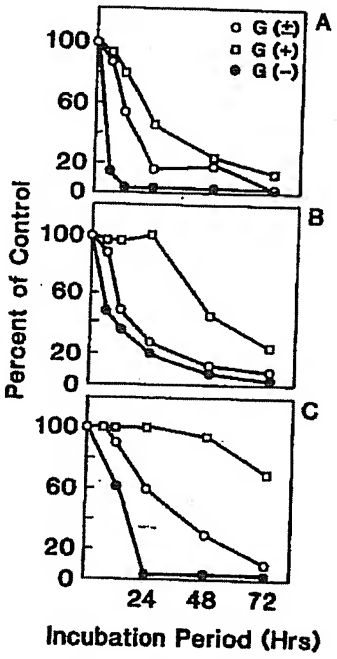

FIG. 2. Time course of the effect of isomers of gossypol on the uptake of [^{3}H]thymidine (A), [^{3}H]leucine (B), and R-123 (C) in OVCA 433 cells. Cells were treated with 5 μg/ml final concentration of the compounds for the indicated time periods. [^{3}H]Thymidine or [^{3}H]leucine (1 μCi) was added for the last 1 hr. For leucine incorporation cells were washed three times with leucine-free medium and then [^{3}H]leucine was added in the same medium. Results are expressed as percentage of controls.

isomer was 3.6 to 12.4 times more active compared to (+) isomer. Racemate exhibited almost half the activity of the equimolar concentrations of (−) isomers. Gossypol acetic acid, the commonly used form of gossypol, was nearly equipotent with the racemate (data not shown). This comparison clearly demonstrates that the activity of gossypol is primarily due to its content of (−)-gossypol. Even though gossypol has been reported to have a selective action on normal reproductive tract of male and female [1], no selectivity was observed toward cancer lines derived from reproductive tract tissues. These results are consistent with other reports about a general *in vitro* antiproliferative effect of gossypol [3–5].

In view of a general antiproliferative action of gossypol, which extended to untransformed fibroblasts as well as mitogen-activated normal lymphocytes, the lack of any reported *in vivo* side effects attributable to such an activity is surprising. It has been suggested that the relative lack of side effects during *in vivo* administration of gossypol as a contraceptive probably reflects a buffering action by serum protein and not any specific tissue or cell distribution. Serum as well as albumin have been demonstrated to inhibit the cytotoxic activity of gossypol [4].

Relative potency of gossypol racemate and isomers was also studied on DNA and protein synthesis and on mitochondrial metabolism, parameters which have been shown to be inhibited by gossypol and have been proposed as possible mechanisms of its activity [2,3,10,11]. It is clear from this study that (−)-gossypol is the most potent and (+)-gossypol the least potent inhibitor of all these three parameters.

Overall the present results demonstrate that gossypol

in vitro acts as a general and nonselective antiproliferative agent and that the antiproliferative and other related activities of gossypol are primarily attributable to its content of (−) isomer.

REFERENCES

1. Qian, S.-Z., and Wang, Z.-C. Gossypol: A potential antifertility agent for males, *Ann. Rev. Pharmacol. Toxicol.* **24**, 329–360 (1984).
2. Maugh, T. H., II. Male "pill" blocks sperm enzyme, *Science* **17**, 314 (1981).
3. Wang, Y.-C., and Rao, P. N. Effect of gossypol on DNA synthesis and cell cycle progression of mammalian cells in vitro, *Cancer Res.* **44**, 35–38 (1984).
4. Haspel, H. C., Ren, Y.-F., Watanabe, K. A., Sonenberg, M., and Corin, R. E. Cytocidal effect of gossypol on cultured murine erythroleukemia cells is prevented by serum protein, *J. Pharmacol. Exp. Ther.* **229**, 218–225 (1984).
5. Tuszynski, G. P., and Cossu, G. Differential cytotoxic effect of gossypol on human melanoma, colon carcinoma, and other tissue culture cell lines, *Cancer Res.* **44**, 768–777 (1984).
6. Tso, W.-W. Gossypol inhibits Ehrlich ascites tumor cell proliferation, *Cancer Lett.* **24**, 257–261 (1984).
7. Waller, D. P., Bunyapraphatsara, N., Martin, A., Vournazos, C. J., Ahmed, M. S., Soejarto, D. D., Cordell, G. A., Fong, H. H. S., Russel, L. D., and Malone, J. P. Effect of gossypol on fertility in male hamsters, *J. Androl.* **4**, 276–279 (1983).
8. Matlin, S. A., Zhou, R., Bialy, G., Blye, R. P., Naqvi, R. H., and Lindberg, M. C. An active male antifertility agent, *Contraception* **31**, 141–149 (1985).
9. Kim, I. C., Waller, D. P., Marcelle, G. B., Cordell, G. A., Fong, H. H. S., Pirkle, W. H., Pilla, L., and Matlin, S. A. Comparative in vitro spermicidal effects of (±)-gossypol, (+)-gossypol, (−)-gossypol and gossypolone, *Contraception* **30**, 253–259 (1984).
10. Johnson, L. V., Walsh, M. L., and Chen, L. B. Localization of mitochondria in living cells with rhodamine 123, *Proc. Natl. Acad. Sci. USA* **77**, 990–994 (1980).
11. Tanphaichitr, N., Chen, L. B., and Bellve, A. R. Direct effect of gossypol on TR-ST cells: Perturbation of rhodamine 123 accumulation in mitrochondria, *Biol. Reprod.* **31**, 1049–1060 (1984).

GYNECOLOGIC ONCOLOGY

AN INTERNATIONAL JOURNAL

Volume 32, Number 3, March 1989

0090-8258/89 $1.50

MADE IN THE UNITED STATES OF AMERICA

GYNECOLOGIC ONCOLOGY

(ISSN 0090-8258)

Published monthly by Academic Press, Inc., 1 East First Street, Duluth, MN 55802

1989: Volumes 32–35. Price: $420.00 U.S.A. and Canada; $500.00 all other countries
All prices include postage and handling.

Information concerning personal subscription rates may be obtained by writing to the Publishers. All correspondence and subscription orders should be addressed to the office of the Publishers at 1 East First Street, Duluth, MN 55802. Send notices of change of address to the office of the Publishers at least 6 to 8 weeks in advance. Please include both old and new addresses.
POSTMASTER: Send changes of address to *Gynecologic Oncology*, 1 East First Street, Duluth, MN 55802.
Second class postage paid at Duluth, MN, and at additional mailing offices.